

# Phylogeny of Hydrothermal-Vent-Endemic Gastropods *Alviniconcha* spp. from the Western Pacific Revealed by Mitochondrial DNA Sequences

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**Abstract.** Mitochondrial genes for cytochrome oxidase I (COI) from hydrothermal-vent-endemic gastropods of the genus *Alviniconcha* were sequenced to determine the phylogenetic relationships among specimens from three areas in the western Pacific. Individuals of *Alviniconcha hessleri* were collected at two vent fields (depths 1470 m and 3600 m) in the Mariana Trough. Specimens collected in the North Fiji Basin could be divided into two genetically distinct groups, both of which also differed from *A. hessleri* from the Mariana Trough. None of the specimens of the genus *Alviniconcha* collected in the Manus Basin differed genetically from the dominant group from the North Fiji Basin. We suggest that the specimens of the genus *Alviniconcha* analyzed in the present study can be tentatively classified into *A. hessleri* and two undescribed species.

## Introduction

Since the late 1970s, various biological communities supported by chemosynthetic energy have been discovered in deep-sea reducing environments, such as hydrothermal vent fields and seep areas (Sibuet and Olu, 1998; Van Dover, 2000). The elucidation of dispersion, isolation, and speciation of endemic species is one of the most important issues of deep-sea biology (Vrijenhoek, 1997). Comparative studies of the genetic structure of their populations will

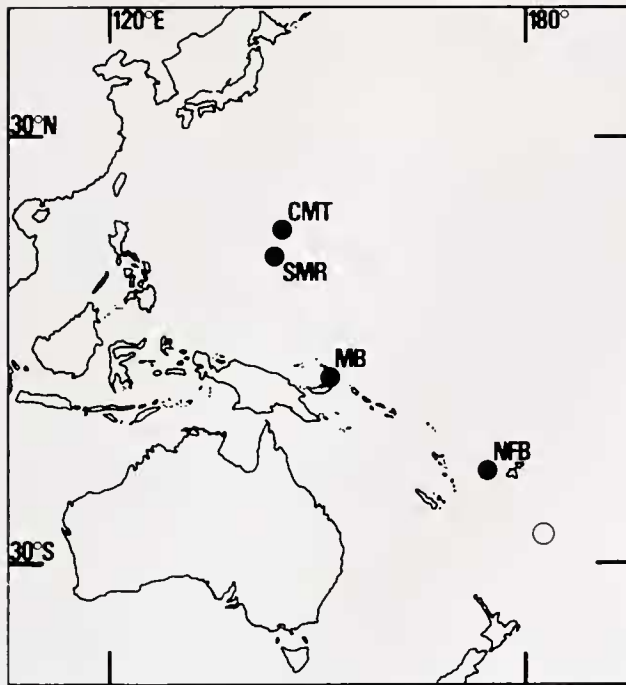
provide useful information about such evolutionary processes.

The population structures of some dominant endemic species in deep-sea reducing environments have been analyzed by electrophoretic examination of allozymes (Vrijenhoek, 1997). Analyses based on DNA sequences would reveal more details of the structures of such populations. To date, population analyses using DNA markers have been reported for just a few species (for review, see Tyler and Young, 1999). In addition, most population genetic studies of organisms in deep-sea reducing environments have been performed on specimens collected in the eastern Pacific and the Atlantic. The accumulation of genetic information about endemic species in deep-sea reducing environments in the western Pacific, where the chemoautotrophy-based communities have unique species composition (Tunnicliffe *et al.*, 1998), should provide more clues to the processes and mechanisms of evolution of fauna in deep-sea reducing environments.

Gastropods of the genus *Alviniconcha*, which live in symbiosis with chemosynthetic bacteria (Stein *et al.*, 1988; Endow and Ohta, 1989), are one of the most dominant groups in the chemoautotrophy-based communities at some hydrothermal vent sites in the western Pacific back-arc basins, namely, the Mariana Trough (Craig *et al.*, 1987; Hessler and Lonsdale, 1991; Fujikura *et al.*, 1997), the Manus Basin (Both *et al.*, 1986; Tufer, 1990; Auzende *et al.*, 1997; Hashimoto *et al.*, 1999), the North Fiji Basin (KAIYO 87 Shipboard Party, 1988; Desbruyères *et al.*, 1994), and the Lau Basin (NAUTILAU Group, 1990; Desbruyères *et al.*, 1994) (Fig. 1).

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**Figure 1.** Collection sites: CMT, Central Mariana Trough; SMR, Southern Mariana Ridge; MB, Manus Basin; NFB, North Fiji Basin. An open circle denotes the location of the Lau Basin.

Okutani and Ohta (1988) described the species *Alviniconcha hessleri* on the basis of specimens collected in the hydrothermal fields along the spreading axis of the central Mariana Trough (3650 m depth). Further populations of *A. hessleri* were discovered in a vent area at a shallower site on the southern Mariana Ridge, at a depth of 1470 m (Johnson *et al.*, 1993). No distinct morphological differences were apparent in the specimens from these two vent sites (Hasegawa *et al.*, 1997).

Populations of *Alviniconcha* gastropods have also been discovered at the hydrothermal vents in the Manus Basin, the North Fiji Basin, and the Lau Basin. On the basis of

slight differences in the radula and the shell, Beck (1991) proposed that *Alviniconcha* in the Manus Basin might be a subspecies of *A. hessleri*. Because distinctive morphological characteristics are scarce, information at the molecular level is necessary to establish exact taxonomic relationships among species of the genus *Alviniconcha*. To date, only one molecular analysis of this genus has been published (Denis *et al.*, 1993). In that study, an electrophoretic analysis of 12 enzymes showed that the populations of *Alviniconcha* gastropods in the North Fiji Basin and the Lau Basin are genetically distinct from one another. Molecular analysis of populations in the other hydrothermal vent sites will provide a useful basis for judgments about the taxonomic status of each population. In the present study, we used nucleotide sequences of mitochondrial DNA to analyze the phylogenetic relationships among populations of the genus *Alviniconcha* in the Mariana Trough, the North Fiji Basin, and the Manus Basin.

### Materials and Methods

During dives of the submersibles *Shinkai 2000* and *Shinkai 6500* of the Japan Marine Science and Technology Center (JAMSTEC) and *Nautille* of Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), 40 specimens of *Alviniconcha hessleri* and 35 of the genus *Alviniconcha* were collected, as summarized in Table 1.

Mitochondrial DNA (mtDNA) was extracted from the deep-frozen head-foot region of each individual by a modified version of the method of Komm *et al.* (1982). In the case of some damaged samples, which had been collected in the North Fiji Basin in 1990 and stored at  $-20^{\circ}\text{C}$ , total DNA was extracted by grinding the tissue, digestion with sodium dodecyl sulfate (SDS) and proteinase K, and extraction with phenol and chloroform.

A fragment (about 450 bp) of the mitochondrial gene for cytochrome oxidase I (COI) was amplified by the polymer-

**Table 1**

Summary of collection information for *Alviniconcha* specimens

Species	Sampling site	Depth (m)	Submersible	Dive #	Sample #
<i>Alviniconcha hessleri</i>	Alice Springs, Central Mariana Trough	3600	<i>Shinkai 6500</i>	D153	CMT-92-1-20
	Forecast Vent, Southern Mariana Ridge	1470	<i>Shinkai 6500</i>	D186	SMR-93-1-11
					D350
<i>Alviniconcha</i> spp.	PACMANUS site, Manus Basin	1630	<i>Shinkai 6500</i>	D914	MB-96-1-20
	White Lady site, North Fiji Basin	1970	<i>Nautille</i>	D12	NFB-90-1-4
			<i>Shinkai 6500</i>	D77	NFB-91-1-4
				D80	NFB-91-5, 6
				D15	NFB-90-5, 6
				D20	NFB-90-7-9
		STARMER II site, North Fiji Basin	1980	<i>Nautille</i>	

ase chain reaction (PCR) with universal metazoan primers, COI-3, 5'-GTNTGRGCNCAAYCAYATRTTYACNGT-3', and COI-6, 5'-GGRTARTCNSWRTANCGNCGNG-GYAT-3' (Shimayama *et al.*, 1990). The conditions for PCR were as follows: 94°C for 60 s; then 30 to 40 cycles at 92°C for 40 s, 40°C for 60 s, and 72°C for 90 s. Genereleaser (BioVenture Inc., Murfreesboro, TN) was used to sequester products of cell lysis that might have inhibited the polymerase. The nucleotide sequences (306 bp) were determined for both strands of PCR products by the dideoxynucleotide chain-termination method using a Sequenase PCR product sequencing kit (United States Biochemical Coop., Cleveland, OH) and primers COI-3 and COI-6.

A longer fragment (about 960 bp) of COI, which contains the region mentioned above, was amplified by PCR using Genereleaser and primers COI-B, 5'-GGATGAACNGT-NTAYCCNCC-3' (Hasegawa *et al.*, 1996) and COI-6. The conditions for PCR were as follows: 94°C for 60 s; then 30 to 40 cycles at 92°C for 40 s, 50°C for 60 s, and 72°C for 90 s. The nucleotide sequences within the upper region of this fragment were determined bidirectionally by a sequencer DSQ-2000L (Shimadzu Corp., Kyoto, Japan) using primers Gastro-3, 5'-TTAGCTGGTGCTTCNTCNATYY-TNGG-3' (Kojima *et al.*, 2000) and TW-2, 5'-ACTACR-TARTANGTRTCRTG-3 (Kojima *et al.*, 1997b). A nucleotide sequence of a single specimen of *A. hessleri* from the South Marina Ridge (No. CMT-92-1) was reported in a previous paper (Kojima *et al.*, 2000). Amino acid sequences of COI were deduced by reference to the modified genetic code of molluscan mtDNA (Shimayama *et al.*, 1990; Hoffmann *et al.*, 1992).

The genetic distances between haplotypes were calculated by Kimura's two-parameter method (Kimura, 1980). Phylogenetic trees were constructed by the neighbor-joining method (Saitoh and Nei, 1987) using MEGA (Kumar *et al.*, 1993) and the maximum parsimony method using the heuristic search approach of the computer program PARSI-MONY, which was provided by Dr. K. Tamura of Tokyo Metropolitan University. *Ifremeria nautilei*, a hydrothermal-vent-endemic gastropod species closely related to *Alviniconcha* (Beck, 1991; Warén and Bouchet, 1993), was used as an outgroup for phylogenetic analysis (Kojima *et al.*, 2000).

Differences in frequencies of haplotypes between populations were examined by the exact test of population differentiation (Raymond and Rousset, 1995) using ARLEQUIN (Schnider *et al.*, 1996) and the randomized chi-squared test of independence (Raff and Bentzen, 1989). Chi-squared values were generated from 1000 simulated random samplings of the data. An unbiased fixation index,  $F_{ST}$  (Weir and Cockerham, 1984), was estimated, and the significance of the indices was tested by a nonparametric

permutation approach using ARLEQUIN (Schnider *et al.*, 1996).

## Results

Partial sequences (696 bp) of mitochondrial genes for COI were determined from 40 specimens of *Alviniconcha hessleri* and 35 specimens of *Alviniconcha* spp. Sequences of all individuals of the genus *Alviniconcha* collected in the North Fiji Basin and the Manus Basin were distinct from those of *A. hessleri* from the Mariana Trough. In addition, the sequences of three specimens of the genus *Alviniconcha* collected during a single dive (Dive 20 of *Nautile*) at the STARMER II site in the North Fiji Basin were very different from those of other specimens from the same area. These other North Fiji Basin specimens included two from the STARMER II site and 10 from the White Lady site. The STARMER II site is only 120 m southwest of the White Lady site in the North Fiji Basin. Individuals with mitochondrial DNA of the dominant type and those with DNA of the less frequent type are referred to hereafter as *Alviniconcha* spp. type 1 and *Alviniconcha* spp. type 2, respectively. Figure 2 shows nucleotide sequences from *Alviniconcha hessleri*, *Alviniconcha* spp. type 1, and *Alviniconcha* spp. type 2. A single amino acid substitution was detected between *Alviniconcha* spp. type 1 and the others (Fig. 2). Within the sequences from each of *A. hessleri*, *Alviniconcha* spp. type 1, and *Alviniconcha* spp. type 2, no amino acid substitutions were detected.

Twenty-seven, thirteen, and three haplotypes were obtained for *A. hessleri*, *Alviniconcha* spp. type 1, and *Alviniconcha* spp. type 2, respectively. Figure 3 shows the phylogenetic relationships among the specimens of the genus *Alviniconcha*. Specimens of *A. hessleri*, *Alviniconcha* spp. type 1, and *Alviniconcha* spp. type 2 formed separate monophyletic clusters. There were greater genetic variations among the three clusters of *Alviniconcha* than within each cluster. The monophyly of *A. hessleri*, *Alviniconcha* spp. type 1, and type 2 was supported by high bootstrap probabilities (93%, 100%, and 100%, respectively). *Alviniconcha hessleri*, *Alviniconcha* spp. type 1, and type 2 formed monophyletic groups in all maximum parsimony trees as well (data not shown).

Three of 27 haplotypes of *A. hessleri* were discovered in the specimens from both the central Mariana Trough and the southern Mariana Ridge (Fig. 3). Statistical analysis, by the exact test of population differentiation (Raymond and Rousset, 1995) and the randomized chi-squared test of independence (Raff and Bentzen, 1989), showed a genetic difference between the populations in the central Mariana Trough and on the southern Mariana Ridge ( $P = 0.035$  and  $P = 0.023$ , respectively). In the case of *Alviniconcha* spp. type 1, four haplotypes were discovered among the specimens from both the North Fiji



Ah ATT ACT GCA GTA TTA TTA TTA CTT TCT TTA OCA GTT TTA GCA GGT GCT ATT ACA ATG CTT TTA ACA GAT OGA  
 A1 ... ..G ... ..R ... ..  
 A2 ... ..Y C. ... ..C ... ..C ... ..C. ... ..

Ah AAT TTY AAT ACT GCT TTC TTT GAC OCA GCT GGA GGT GGT GAT OCA ATT TTA TAY CAA CAT TTA TTT TGA TTC  
 A1 ... ..C ... ..T ..R ..Y ..R ... ..C ..G ... ..  
 A2 ... ..C ... ..G ... ..C ..T ... ..C ... ..C. ... ..

Ah TTT GGG CAC OCA GAA GTT TAT ATT TTA ATT CTT CCT GGC TTT GGA ATG ATT TCT CAT ATT GTT AGA CAT TAT  
 A1 ..Y ... ..R ... ..C ... ..  
 A2 ... ..T ... ..T ... ..A ... ..C ... ..C ... ..

Ah TCA GCY AAG AAA GAA ACY TTT GGG ACA CTT GGA ATA ATY TAT GCA ATG ATA GCA ATT GGT TTA TTA GGT TTT  
 A1 ... ..T ... ..T ... ..G ..Y ... ..A ... ..C. ... ..  
 A2 ... ..T ... ..T ... ..A ... ..G ..T ... ..A ..G ... ..C. ... ..

Ah ATT GTA TGA GCT CAY CAT ATG TTT ACA GTA GGT ATG GAT GTA GAT ACT OGR GCT TAY TTT ACA GCA GOC ACT  
 A1 ... ..T ... ..A ... ..T ..C ... ..T ... ..  
 A2 ... ..C ... ..A ... ..T ... ..

Ah ATA ATT ATT GCY GTT OCA ACT GGA ATT AAG GTY TTC AGY TGA CTY GCC ACA ATT CAT GGS TCA AAA ATC AAG  
 A1 ... ..Y ... ..T ... ..T ... ..T ..Y ... ..C ..A ... ..T ..R  
 A2 ... ..T ... ..T ... ..T ... ..T ... ..Y ... ..A ... ..T ... ..

Ah TAT GAA ACT CCT ATG CTT TGA GCT TTA GGR TTT ATT TTC TTG TTT ACT GTA GGG GGT TTA ACT GGA ATT GTT  
 A1 ... ..G ... ..Y C. ... ..  
 A2 ... ..A ..C ... ..A ... ..A ..A ... ..

Ah CTT TCT AAT TGY TCY YTA GAT ATT ATG ATG CAY GAY ACT TAC TAT GTT GTA GCY CAT TTC CAT TAC GTR CTA  
 A1 ... ..C ..T C. ... ..T. ..C ..C ... ..T ..C ... ..G ..R  
 A2 ... ..T ..T T. ... ..T ..T ... ..T ... ..C ... ..T ..T T..

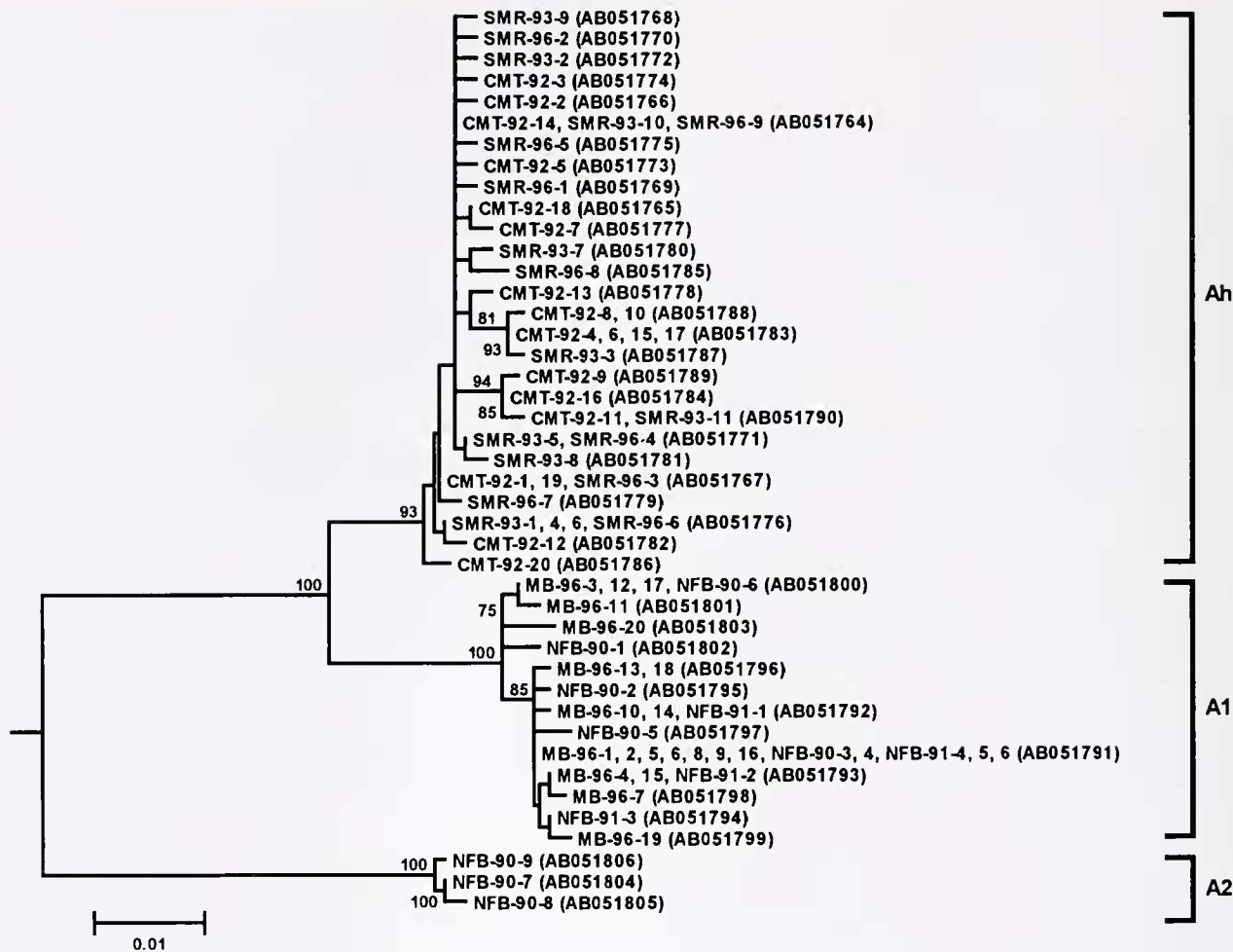
Ah TCA ATR GGR GCA GTC TTY GCC YTA TTT GCA GCT TTT AAC TAT TGA TTY OCA TTA ATR AGA GGG GTA ACA CTT  
 A1 ... ..G ..A ... ..C ..Y T. ... ..Y ... ..C ... ..G ..R ... ..  
 A2 ... ..A ..A ..G ... ..T ..T C. ... ..T ..C ... ..C ... ..G ..G ... ..

Ah CAC TCT CGT TGA ACA AAA GCT CAT TTT TAT ATY ATR TTT ATY GGR GTA  
 A1 ... ..Y ..G ... ..T ..R ... ..  
 A2 ..T ... ..A ... ..C ... ..T ..G ... ..T ..A ... ..

**Figure 2.** Nucleotide sequences of mitochondrial genes for cytochrome oxidase I from *Alviniconcha hessleri* (Ah), *Alviniconcha* spp. type 1 (A1), and *Alviniconcha* spp. type 2 (A2). Dots indicate nucleotides identical to those in Ah. An underlined codon encodes an amino acid different from that in Ah. R denotes G or A; Y is T or C; S is G or C.

Basin and the Manus Basin (Fig. 3), and there was no genetic difference between the two populations ( $P = 0.793$  for the exact test of population differentiation, and

$P = 0.705$  for the randomized chi-squared test of independence). Although the  $F_{ST}$  value between the two populations of *A. hessleri* was significant ( $P < 0.05$ ) and



**Figure 3.** Phylogenetic tree for specimens of *Alviniconcha* spp. The tree was constructed by the neighbor-joining method with *Ifremeria nautilei* (the most dominant haplotypes in the populations of the Manus Basin and the North Fiji Basin) as an outgroup. Numbers refer to specimens indicated in Table 1. Bootstrap values are shown above or below branches of clades that are supported by bootstrap values of more than 70%. Ah, A1, and A2 denote *Alviniconcha hessleri*, *Alviniconcha* spp. type 1, and *Alviniconcha* spp. type 2, respectively. The nucleotide sequence of each specimen will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under the accession number in parentheses.

estimated to be 0.047, that of *Alviniconcha* spp. type 1 was not significant ( $P > 0.05$ ).

### Discussion

In the present study, we analyzed the phylogenetic relationships among populations of the *Alviniconcha* gastropods in hydrothermal areas in the western Pacific. Both the neighbor-joining (NJ) method and the maximum parsimonious (MP) method showed that the 43 haplotypes identified among 80 specimens of the genus *Alviniconcha* formed three monophyletic clusters (Fig. 3). In the NJ tree, the monophyly of each of the three clusters was supported by a high bootstrap probability. The three clusters of *Alviniconcha* were distinct from one another, with a smaller range of

sequence variations within clusters than among clusters. Therefore, we suggest tentatively that the specimens of the genus *Alviniconcha* analyzed in the present study should be classified as *A. hessleri* and two undescribed species.

Although the habitat on the southern Mariana Ridge is much shallower than the type locality of *A. hessleri* (the central Mariana Trough), all haplotypes identified from specimens collected at two vent sites formed a single cluster (Fig. 3). Although the exact test of population differentiation, a randomized chi-squared test, and the test of significance of the  $F_{ST}$  value showed that a genetic difference between the two populations was significant at the 5% level, they shared three haplotypes. From the pairwise  $F_{ST}$  value, the absolute number of migrants exchanged between these

two populations was estimated to be 10.2. These results suggest that these two populations are conspecific and might be connected by significant gene flow. The present results are consistent with the absence of distinct morphological differences between these populations (Hasegawa *et al.*, 1997). The distribution of conspecific populations in the bathyal zone and the abyssal zone is in marked contrast with the strict depth zonation exhibited by endemic groups that inhabit the seep areas off central Japan, namely, bivalves of the genus *Calyplogena* (Kojima and Ohta, 1997) and vestimentiferans (Kojima *et al.*, 1997b). This difference might be due to environmental factors, such as water currents and the topography of the sea floor, or to differences in biological characteristics among groups.

The absence of genetic differences between the populations of *Alviniconcha* spp. type 1 in the Manus Basin and in the North Fiji Basin provides a typical example of the active interchange of fauna between western Pacific back-arc basins, which was first noted by Hessler and Lonsdale (1991). For *Ifremeria nautilei*, which is related to *Alviniconcha* spp., Kojima *et al.* (2000) reported that no haplotype was shared between the Manus Basin and the North Fiji Basin and concluded that the two populations should be considered to be conspecific populations that are isolated geographically from one another. The morphology of the larval shell of *Alviniconcha* is suggestive of planktonic development, whereas the larval type of *Ifremeria* remains unclear (Warén and Bouchet, 1993). The difference in degree of genetic differentiation between *I. nautilei* and *Alviniconcha* spp. type 1 might be attributed to differences in the larval type (Kojima *et al.*, 2000).

Denis *et al.* (1993) reported significant genetic differences between a population of *Alviniconcha* in the North Fiji Basin and one in the Lau Basin, and they proposed that individuals in these two populations should be treated as separate species. The North Fiji Basin is situated near the Lau Basin (Fig. 1); thus *Alviniconcha* spp. type 2 might be conspecific with the Lau Basin species proposed by Denis *et al.* (1993). Unfortunately, samples from the Lau Basin are not available, so we have been unable to test this hypothesis. To clarify the taxonomic status of these putative species, detailed morphological examination and further molecular analysis are required.

The dominance of gastropods of the family Provannidae offers one of the prominent features in some chemoautotrophic communities in the back-arc basins in the western Pacific. This study and our previous ones (Kojima *et al.*, 2000) revealed the population structures of some species in the two representative genera of this family, namely *Alviniconcha* and *Ifremeria*. Although many other endemic taxa have been reported from the western Pacific (Desbruyères and Segonzac 1997; Miura *et al.*, 1997; Okutani *et al.*, 1999; Fujikura *et al.*, 2000), information about their population structures is still limited (Kojima *et al.*,

1997a, b). Information about the population structures of many endemic species in deep-sea reducing environments will be needed to reveal details of the evolution in such environments in the western Pacific and to compare them with those in other oceans.

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